Serine protease inhibitors modulate chemotactic cytokine production by human lung fibroblasts in vitro

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1Research Service, Southern Arizona Veterans Health Care System, and Arizona Respiratory Center, University of Arizona, Tucson, Arizona 85723; and 2The First Department of Internal Medicine and 3The Second Department of Surgery, School of Medicine, Shinshu University, Matsumoto, Japan 390-0802
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Numanami, Hiroki, Sekiya Koyama, Esturo Sato, Masayuki Haniu, Dan K. Nelson, Jeffrey C. Hoyt, Jon L. Freels, Michael P. Habib, and Richard A. Robbins. Serine protease inhibitors modulate chemotactic cytokine production by human lung fibroblasts in vitro. Am J Physiol Lung Cell Mol Physiol 284: L882–L890, 2003. First published December 13, 2002; 10.1152/ajplung.00211.2002.—Chemotactic chemokines can be released from lung fibroblasts in response to interleukin (IL)-1β and tumor necrosis factor (TNF)-α. An imbalance between proteases and anti-proteases has been observed at inflammatory sites, and, therefore, protease inhibitors might modulate fibroblast release of chemotactic cytokines. To test this hypothesis, serine protease inhibitors (FK-706, α1-antitrypsin, or Na-p-tosyl-l-lysine chloromethyl ketone) were evaluated for their capacity to attenuate the release of neutrophil chemotactic activity (NCA) or monocYTE chemotactic activity (MCA) from human fetal lung fibroblasts (HFL-1). Similarly, the release of the chemotactants IL-8, granulocyte colony-stimulating factor, monocyte chemotactant protein-1, macrophage colony-stimulating factor, and granulocyte/macrophage colony-stimulating factor, from HFL-1, were evaluated in response to IL-1β and TNF-α. NCA, MCA, and chemotactic cytokines were attenuated by FK-706. However, matrix metalloproteinase inhibitors were without effect, and cysteine protease inhibitors only slightly attenuated chemotactic or cytokine release. These data suggest that IL-1β and TNF-α may stimulate lung fibroblasts to release NCA and MCA by a protease-dependent mechanism and that serine protease inhibitors may attenuate the release.

neutrophil; monocyte; interleukin-8; monocyte chemotactant protein-1; granulocyte/macrophage colony-stimulating factor

A BROAD SPECTRUM OF INFLAMMATORY lung disorders is characterized by tissue destruction and remodeling, caused by an excess of protease activity, e.g., adult respiratory distress syndrome, pulmonary emphysema, bronchopulmonary dysplasia, and cystic fibrosis (2, 3, 48). Protease inhibitors may protect the lung from detrimental destruction by proteases. However, anti-proteases may also modulate neutrophil migration (22, 23, 40). This observation suggests that protease inhibitors not only might act directly on proteolysis but may also attenuate inflammation by inhibiting production of chemokines.

The fibroblast is the principal connective tissue cell involved in the synthesis of the collagenous and non-collagenous components of the extracellular matrix. This synthetic activity serves an important structural function by providing a frame network for organ integrity. In addition to this traditionally accepted function, recent studies have demonstrated that fibroblasts may also participate in the orchestration of acute and chronic inflammation. In this context, fibroblasts release monocyte chemoattractant protein-1 (MCP-1), granulocyte/macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-β, in response to inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (25, 26, 28, 33). Moreover, fibroblasts secretion a variety of proteases, including plasmin (10), fibroblast activation protein (37), tissue-type plasminogen activator (46), urokinase-type plasminogen activator (16), and a calcium-dependent serine protease (34). Therefore, the fibroblast, because of its anatomical location and protease secretion, is in a pivotal position to participate in and direct communications between interstitial and vascular events in pulmonary inflammation and fibrosis.

On the basis of the rationale above, the purpose of this study was to demonstrate that protease inhibitors modulate the release of proinflammatory cytokines by HFL (human fetal lung)-1 cells. The results demonstrate that the serine protease inhibitor FK-706 inhibited the release of neutrophil chemotactic activity (NCA) and monocyte chemotactic activity (MCA) from the lung fibroblast cell line, HFL-1. These results suggest that the interaction between HFL-1 and proinflammatory cytokines involves proteolytic mechanism(s) and that protease inhibitors may have the potential for modulating lung inflammation.

MATERIALS AND METHODS

Cell cultures. HFL-1 fibroblasts (lung, diploid, human, passage 14) were purchased from American Type Culture

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Collection (Rockville, MD). The HFL-1 cells were cultured, according to previously described methods, in Ham’s F-12 medium with 10% heat-inactivated fetal bovine serum (24, 35, 36). After 2–4 days in culture, the cells had reached confluence and were then used for experiments.

Protease inhibitors and stimulants. FK-706 (C₉H₁₄F₇F₅N₂NaO₇; Fujisawa Pharmaceutical, Osaka, Japan) was used as a serine protease inhibitor (38). HFL-1 cells were exposed to human recombinant IL-1β (500, 50, 5, and 0.5 pg/ml; Sigma, St. Louis, MO) or TNF-α (100, 10, 1, and 0.1 ng/ml; Sigma) at 37°C in a humidified 5% CO₂ atmosphere for 24 h. In some experiments, HFL-1 cells were pretreated with several concentrations of the serine protease inhibitors FK-706 (50, 10, and 5 μg/ml), α1-antitrypsin (200, 100, and 10 μg/ml; Sigma), or Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK; 200 and 100 μM; Sigma). In the other experiments, the cysteine protease inhibitor, leupeptin (200 and 100 μM; Sigma), or a metalloprotease inhibitor of neutral endopeptidase 24.11, phosphoramidon (200 and 100 μM; Sigma), were used. The concentrations of FK-706 were based on results showing that neutrophil elastase-induced lung hemorrhage of mice was significantly inhibited by intratracheal treatment with FK-706 at a dose from 1 to 100 μg (38). In addition, the concentrations of other protease inhibitors were based on previous studies (22, 23). In some cultures, neutrophil elastase (Elastin Products, Owingsville, MO) was used to reverse the effects of FK-706. IL-1β and TNF-α were tested for LPS contamination, and LPS was shown to be <0.1 ng/ml. These cytokines did not cause HFL-1 cell injury (no deformity of cell shape, no detachment from culture dish, and >98% viability by trypan blue exclusion) after 24 h of incubation at the highest concentration used. The culture supernatant fluid was harvested and frozen at −80°C until assay. At least six separate HFL-1 cell supernatant fluids were harvested from cultures for each experimental condition.

Measurement of cytokines in the supernatant fluids. The concentrations of IL-8, MCP-1, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and GM-CSF were measured in the cell supernatant fluids using commercially available ELISAs (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions, in duplicate. The minimum concentrations detected by these methods were 10 pg/ml for IL-8, 5.0 pg/ml for MCP-1, 9.0 pg/ml for M-CSF, 20 pg/ml for G-CSF, and 3.0 pg/ml for GM-CSF.

Effects of protease inhibitors on NCA and MCA by HFL-1 supernatant fluids. Polymorphonuclear leukocytes were purified from heparinized normal human blood by the method of Böyum (4). The resulting cell pellet consisted of >96% neutrophils and >98% viable cells as determined by trypan blue and erythroin exclusion. The cells were suspended in Gey’s balanced salt solution (GBCO, Grand Island, NY) containing 2% bovine serum albumin (BSA; Sigma) at pH 7.2 to give a final concentration of 3.0 × 10⁶ cells/ml. This suspension was used for the neutrophil chemotaxis assay.

Mononuclear cells for the chemotaxis assay were obtained from normal human volunteers by Ficoll-Hypaque density centrifugation to separate red blood cells and neutrophils from mononuclear cells. The preparation routinely consisted of 30% large monocytes and 70% small lymphocytes determined by morphology and α-naphthyl acetate esterase staining (Sigma), with >98% viability as assessed by trypan blue and erythroin exclusion. The cells were suspended in Gey’s

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**Fig. 1.** Neutrophil chemotactic activity (NCA; A and C) and monocyte chemotactic activity (MCA; B and D) in response to IL-1β (A and B) or TNF-α (C and D) from human fetal lung fibroblasts (HFL-1) monolayers after 24 h of incubation (n = 6). Chemotactic activities are on the ordinate, and the concentration of IL-1β and TNF-α is on the abscissa. Values are expressed as means ± SD. *P < 0.05 compared with supernatant fluids without stimuli. **P < 0.01 compared with supernatant fluids from HFL-1 cells cultured without stimuli. ***P < 0.001 compared with supernatant fluids from HFL-1 cells cultured without stimuli.
balanced salt solution containing 2% BSA at pH 7.2 to give a final concentration of 5.0×10^6 cells/ml. The suspension was then used for the monocyte chemotaxis assay.

The chemotaxis assay was performed by a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD), as has been described previously (13). A 10-mm-thick polyvinylpyrrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA) with a pore size of 3 μm for neutrophil chemotaxis and 5 μm for monocyte chemotaxis was placed over the bottom wells. The silicon gasket and top pieces of the chamber were applied, and 50 μl of the cell suspension was placed into the top wells above the filter. The chambers were incubated in humidified air in 5% CO₂ at 37°C for 30 min for neutrophil chemotaxis and 90 min for monocyte chemotaxis. After incubation, the chamber was disassembled, and nonmigrated cells were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik (American Scientific Product, McGaw Park, IL), and

### Table 1. Concentration of cytokines in HFL-1 culture supernatant fluids after 24 h

<table>
<thead>
<tr>
<th></th>
<th>IL-8, 100 pg/ml</th>
<th>TNF-α, 1 ng/ml</th>
<th>Control</th>
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<tbody>
<tr>
<td></td>
<td>Without FK-706</td>
<td>With FK-706</td>
<td>Without FK-706</td>
</tr>
<tr>
<td>IL-8</td>
<td>227.4 ± 41.6</td>
<td>87.3 ± 23.4†</td>
<td>475.9 ± 80.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>429.2 ± 59.1</td>
<td>276.7 ± 19.0α</td>
<td>924.6 ± 96.7</td>
</tr>
<tr>
<td>M-CSF</td>
<td>61.5 ± 1.4</td>
<td>50.5 ± 5.3α</td>
<td>135.7 ± 8.3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1,709 ± 167</td>
<td>1,426 ± 96α</td>
<td>1321 ± 294</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>84.3 ± 4.9</td>
<td>55.6 ± 5.9α</td>
<td>422.1 ± 59.7</td>
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</table>

Values are means ± SD (pg/ml), n = 4. Human fetal lung fibroblasts (HFL-1) were cultured with IL-1β or TNF-α and with or without FK-706 (10 μg/ml). IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor. *P < 0.01 compared with HFL-1 cells cultured without FK-706. †P < 0.001 compared with HFL-1 cells cultured without FK-706.

Fig. 2. Effects of FK-706 on IL-8 (A and B) or monocyte chemoattractant protein-1 (MCP-1; C and D) stimulated with IL-1β (100 pg/ml, A and C) or TNF-α (1,000 pg/ml, B and D) from HFL-1 monolayers after 24 h of incubation (n = 4). The concentration of IL-8 and MCP-1 is on the ordinate, and experimental groups are on the abscissa. Values are expressed as means ± SD. **P < 0.01 compared with supernatant fluids from HFL-1 cells cultured with IL-1β or TNF-α. ***P < 0.001 compared with supernatant fluids from HFL-1 cells cultured with IL-1β or TNF-α.
mounted on a glass slide. Cells that completely migrated through the filter were counted by using light microscopy in 10 random high-power fields per well.

**Evaluation of mRNA expression.** Cytokine mRNA was analyzed by RT-PCR. HFL-1 cells were incubated with FK-706 and cytokines for 12 h, and total cellular RNA was extracted from adherent cells using a modification of the methods of Chomczynski and Sacchi (7). The RNA was reverse transcribed using a commercially available kit (Promega, Madison, WI). One microgram of the reverse-transcribed DNA was then mixed with Ready to Go PCR Beads (Pharmacia, Piscataway, NJ), and the front and back primers, using a commercially available primer pair (R&D Systems), were added at a final concentration of 0.3 μM. PCR was performed in a Perkin Elmer 480 thermal cycler using 94°C for 2 min and 26 cycles consisting of 94°C for 45 s, primer annealing at 55°C for 45 s, and primer extension at 72°C for 45 s, followed by 72°C for an additional 7 min. β-Actin was used as a "housekeeping gene" with PCR. The DNA was subjected to agarose gel, and the intensity of the bands quantified by densitometry. The results were expressed as the ratio of intensity to the β-actin.

**Statistical analysis.** Data were analyzed by Dunnett's one-way analysis of variance with a Fisher's protected least significant differences test. In all cases, P < 0.05 was considered significant. The data are expressed as means ± SD.

**RESULTS**

**Release of NCA and MCA from HFL-1 cells in response to IL-1β and TNF-α.** IL-1β or TNF-α stimulated the release of NCA and MCA from HFL-1 in a dose-dependent fashion (Fig. 1, A–D, n = 6). The release of NCA and MCA was observed after 24 h of incubation in response to 100 pg/ml of IL-1β and 1 ng/ml of TNF-α. IL-1β and TNF-α induced the release of significant NCA at 5 pg/ml and 0.1 ng/ml, and MCA at 0.1 pg/ml and 0.1 ng/ml, respectively.

**Effects of FK-706 on cytokine production from HFL-1.** HFL-1 spontaneously released IL-8, MCP-1, M-CSF, G-CSF, and GM-CSF, but the inflammatory cytokines IL-1β and TNF-α stimulated the release of these cytokines from HFL-1 (Table 1, n = 4).

The release of IL-8 and MCP-1 was dose dependently inhibited by FK-706 (Fig. 2, n = 4). FK-706 had no stimulating effect on release of these chemokines from HFL-1 (Table 1).

FK-706 also inhibited the release of M-CSF, GM-CSF, and G-CSF in response to IL-1β and TNF-α from HFL-1 cell monolayers (Table 1, n = 4).
Effects of protease inhibitors on NCA and MCA. IL-1β or TNF-α stimulated NCA and MCA from HFL-1. FK-706 inhibited, in a dose-dependent manner, NCA and MCA from HFL-1 stimulated with IL-1β or TNF-α (Fig. 3, n = 6). FK-706 alone had no effect on baseline release of NCA and MCA under control conditions (P > 0.05). The other serine protease inhibitors α1-antitrypsin and TLCK reduced the IL-1β- or TNF-α-induced NCA and MCA (Fig. 4, n = 6). None of these inhibitors altered baseline NCA and MCA release (P > 0.05, Fig. 4).

IL-1β- and TNF-α-induced release of NCA and MCA from HFL-1 monolayers was not modulated by phosphoramidon at all concentrations tested (P > 0.05, Fig. 5, A and B, n = 6). However, leupeptin significantly inhibited the release of NCA and MCA (Fig. 5, C and D, n = 6).

Effect of elastase on the anti-inflammatory action of FK-706. Neutrophil elastase attenuated the anti-inflammatory action of FK-706. In the sample of preincubated FK-706 and 50 µg/ml of neutrophil elastase, the inhibition for releases of NCA, MCA, and IL-8 in response to IL-1β decreased compared with FK-706 (Fig. 6, n = 4). The preincubated reagents alone had no effect on baseline releases of NCA, MCA, and IL-8 under control conditions (P > 0.05).

Effects of FK-706 on mRNA expression from HFL-1. Semiquantitative RT-PCR was performed to evaluate the effect of FK-706 on cytokine mRNA expression in HFL-1 (n = 3). IL-1β- or TNF-α-induced IL-8 and MCP-1 mRNA expressions were suppressed by pretreatment with FK-706 (Fig. 7).

**DISCUSSION**

In the present study, we demonstrated that HFL-1 released NCA, MCA, and cytokines, including IL-8, MCP-1, G-CSF, M-CSF, and GM-CSF, in response to proinflammatory cytokines. Several structurally different serine protease inhibitors attenuated the release of

![Fig. 4. Effects of α1-antitrypsin (α1-AT; A and B) and Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK; C and D) on NCA (closed bars) and MCA (open bars) in response to IL-1β (A and C) or TNF-α (B and D) from HFL-1 monolayers after 24 h of incubation (n = 6). NCA and MCA are on the ordinate, and the experimental groups are on the abscissa. Values are expressed as means ± SD. **P < 0.01 compared with supernatant fluids from HFL-1 cells cultured with IL-1β or TNF-α. ***P < 0.001 compared with supernatant fluids from HFL-1 cells cultured with IL-1β or TNF-α.](chart)
NCA and MCA by the HFL-1 in response to IL-1β or TNF-α. FK-706 suppressed the expression of these cytokines. These protease inhibitors had no effect on NCA or MCA by unstimulated HFL-1. Consistent with these results, FK-706 treatment of HFL-1 also showed the suppressive effect on cytokine production and expression of IL-8 and MCP-1 mRNA. These results suggest that HFL-1 interaction with IL-1β or TNF-α may involve proteolytic mechanisms, and serine protease inhibitors may modulate the interaction between proinflammatory cytokines and HFL-1.

Protease imbalance has been proposed in the pathogenesis of several acute and chronic inflammatory diseases, including pulmonary emphysema (5, 12), adult respiratory distress syndrome (27, 31), cystic fibrosis (17, 32), chronic bronchitis (29), septic shock (44), and other inflammatory states (1, 14). Antiproteases have been reported to modulate neutrophil migration in response to several stimuli (22, 23, 40). Furthermore, Churg and coworkers (8) noted that α1-antitrypsin suppressed silica-induced neutrophil influx and MCP-1 gene expression. Kikuchi et al. (19) reported that secretory leukocyte protease inhibitor (SLPI) and α1-antitrypsin augmented hepatocyte growth factor production in human lung fibroblasts. SLPI has also been suggested to suppress prostaglandin E2 and metalloproteinase production in monocytes (49). In this context, protease inhibitors may reduce lung injury directly by preventing destruction of connective tissue, but also indirectly by attenuating recruitment of neutrophils and monocytes to sites of inflammation. Activated inflammatory cells release a variety of degradative enzymes, oxygen metabolites, and cytokines, which may lead to further tissue damage.

We investigated the effect of protease inhibitors on HFL-1 because lung fibroblasts constitute 35–40% of the cells in the interstitium of the lung and are activated to proliferate and synthesize various cytokines during inflammation (24). Moreover, fibroblasts have been reported to produce large amounts of the chemotactic cytokines, IL-8, MCP-1, G-CSF, and GM-CSF, in response to various stimuli (41, 42). In the present context, protease inhibitors may reduce lung injury directly by preventing destruction of connective tissue, but also indirectly by attenuating recruitment of neutrophils and monocytes to sites of inflammation. Activated inflammatory cells release a variety of degradative enzymes, oxygen metabolites, and cytokines, which may lead to further tissue damage.

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study, IL-1β or TNF-α stimulated the release of these cytokines and an increase in NCA and MCA. These observations are consistent with the concept that fibroblasts may be an important source of neutrophil and monocyte chemoattractants in lung inflammation.

The present study confirmed that fibroblasts had the potential for contributing to airway inflammation by releasing NCA and MCA and suggested that an imbalance between protease and antiprotease activity in the lower respiratory tract might augment lung inflammation by modulating the responsiveness of fibroblasts. However, a limitation of these studies was that they were done in vitro with a human fibroblast cell line. The effects of protease inhibitors on primary cultures of human airway fibroblasts and demonstrating this phenomenon in vivo are important issues for future research.

In this study we used phosphoramidon as a matrix metalloprotease inhibitor, but it did not inhibit either NCA or MCA. Other reports indicate that matrix metalloprotease inhibitors reduce inflammation in vivo and in vitro (18, 43). Phosphoramidon is a selective inhibitor of neutral endopeptidase (NEP), a metalloprotease on the surface membrane of fibroblasts (15). Moreover, NEP expression from lung fibroblasts was enhanced by IL-1β and TNF-α (21). In contrast, other reports suggest that NEP may reduce inflammation by enzymatic cleavage of inflammatory substances, such as bradykinin (45), substance P (39), and the chemoattractant peptide formyl-Met-Leu-Phe (11). These later observations would suggest that matrix metalloproteinase inhibitors are unlikely to work as anti-inflammatory agents by inhibition of NEP, whose activity may reduce inflammation.

FK-706 is a water-soluble, chloromethyl ketone derivative that inhibits serine proteases (38). The $K_i$ value for human neutrophil elastase is $4.2 \text{ nM}$. This compound inhibits human neutrophil elastase activity and porcine pancreatic elastase activity with respective $IC_{50}$ values of 83 and 100 nM. FK-706 acted against elastase-induced lung hemorrhage and elastase-induced skin edema in animal models (38). FK-706 consists of a trifluoromethyl ketone motif, as an active site, with a molecular mass of 0.59 kDa. Although it is not fully understood which signaling pathways were stimulated and/or inhibited in response to protease inhibitors, chloromethyl ketone derivatives reduce activation of NF-κB, a major nuclear factor inducing the release of several inflammatory cytokines (6, 20). In this study, we demonstrated that FK-706 blocked the release of inflammatory cytokines and suppressed the expression of IL-8 and MCP-1 mRNA. These data indicate that FK-706 may attenuate the response of lung fibroblasts to IL-1β and TNF-α upstream at or before transcription, possibly through suppression of NF-κB signaling pathway.

Although most serine protease inhibitors cannot penetrate cell membranes, the present study revealed that protease inhibitors affected the responses of HFL-1 cells to proinflammatory cytokines. The mechanisms of antiprotease inhibition of the inflammatory action, including receptors and signaling pathways, are still unclear. In recent years, protease-activated receptors have been described that are proteolysis-activated G protein-coupled receptors (9, 30). Neutrophil elastase induces IL-8 gene upregulation in bronchial epithelial cells through an IL-1β receptor-associated kinase signaling pathway, suggesting that neutrophil elastase stimulates an as yet unidentified receptor (47). In this study, neutrophil elastase attenuated the anti-inflammatory action of FK-706. In this context, FK-706 may act through protease-activated receptors or other unidentified mechanisms.

In conclusion, a number of protease inhibitors attenuated NCA and MCA in response to proinflammatory
cytokines on HFL-1. Therefore, proteolytic activity appears to play a critical role in the release of NCA and MCA. Antiprotease attenuated this activity at the transcriptional level. Thus extracellular protease inhibitors, such as FK-706, may be effective in attenuating inflammatory interaction between HFL-1 and proinflammatory cytokines.

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REFERENCES


